CHARACTERIZATION OF CIRCADIAN RHYTHMS IN THE MOUSE INTESTINE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Characterization of Circadian Rhythms in the Mouse Intestine

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Circadian clocks drive rhythmic gene expression to enable biological functions to perform optimally at the most appropriate time of day. Circadian rhythms rely on the binding of the transcription factor CLOCK:BMAL1 to specific DNA binding sites, causing both tissue-specific and time-specific rhythmic gene expression and protein synthesis. It is biologically efficient to have tissue-specific rhythm for tissue-specific functions, such as digestion within the small intestine. Physiological rhythms in the small intestine have been well described, but it is still unclear how the molecular clock regulates gene expression at the genome wide level. In my project, I will set up techniques to efficiently determine rhythmicity in the intestine through RNA extraction and mRNA analysis using quantitative PCR. I will also determine the genes targeted by CLOCK:BMAL1 using the chromatin immunoprecipitation (ChIP) method. This protocol will then be used to characterize the genes that are rhythmically expressed in different regions of the intestinal tract through quantitative real-time PCR. Completion of my project will enable the lab to examine more precisely how the circadian clock controls the rhythmic digestion in mammals, and will represent a strong foundation for further studies that will examine the role of rhythmic feeding behavior in the biology of the intestine.

CHAPTER I

INTRODUCTION

Biological rhythms dictate virtually all organisms, ranging from bacteria, fungi, and plants to humans, quite literally day in and day out with sleep-wake cycles and hormonal and homeostatic functions (1,8). Studies show that circadian clocks are important to drive biological functions because they allow for optimal tissue-specific function at the most appropriate time of the day. Circadian rhythms are also the driving force behind sleep, reproduction, and migration is some animals (7). In the modern world of global travel, technology, and 24/7 stores, circadian rhythms are now more important than ever. Although the clocks can adjust to local time based on light signals (7), imbalances in this rhythm are what cause jet lag and more serious circadian rhythm sleep disorders (CRSDs) (8). Also, with about 15% to 30% of the working population involved in shift work that can disturb natural circadian rhythm cycles, it is shown to have an influence on sleep, performance, mood, and even heart rate (6). Circadian rhythm imbalances have a direct involvement in the development of neuropsychiatric disorders including depression, anxiety, schizophrenia, bipolar disorder, drug abuse, obesity, and others (1,3,8). More specifically related to this project, irregular rhythmicity in shift workers and intercontinental travels show a decrease in intestinal functions like nutrient absorption and can worsen irritable bowel syndrome.

The molecular clock's target genes have DNA-specific binding sites that attract the heterodimer CLOCK:BMAL1, which binds to repressor core clock genes Period (*Per1, Per2*) and Cryptochrome (*Cry1, Cry2*) allowing their transcription. The feedback repressor genes are then

translocated to the nucleus to inhibit the transcriptional activity of CLOCK:BMAL1. The gradual degradation of PER and CRY reactivates the transcription of CLOCK:BMAL1, completing the cyclic feedback loop and activating a new round of rhythmic transcription. This feedback loop, along with other signaling proteins, initiates and promotes the rhythmic biochemical and metabolic pathways through protein synthesis on an individual cell level through other feedback loops (7). My project focuses specifically on characterizing clock controlled circadian rhythm genes within the intestine. The small intestine has been shown to be rhythmic along with the other intestinal tissues such as the colon, although not always with the same magnitude of gene expression (6,8). The rhythmicity of the intestinal tissues has not been well characterized. The goal of the project is to set the path for more in-depth experiments with the knowledge, expertise, and data that these experiments will provide. Knowing that we have the ability to successfully classify and map the circadian rhythm in this specific tissue sub-set will allow the Menet lab to confidently proceed to more in-depth experiments that alter different aspects of feeding rhythmic behavior and other variables, with this data supporting those claims as the control.

CHAPTER II

OBJECTIVE AND METHODOLOGY

The objective of my project is to both set up an efficient and reliable protocol to successfully characterize the genes that are rhythmically targeted by CLOCK:BMAL1 in the intestine using mice as the model and to quantify the steady-state levels of mRNA expression at different time points. Tissue samples from the intestine of the facility mice that have been adapted to the correct light cycle will be used. Wild type (WT) (C57BL/6 strain) mice are used in this lab and will be used in this experiment because of their wide use throughout circadian rhythm studies. The experiment will take place using four-hour blocks of time available for tissue harvesting using Zeitgeber Time (ZT) from ZT2 to ZT22, where ZT2 through ZT10 are the light on cycle and ZT14-ZT22 are light off cycle. By convention, ZT0 corresponds to "light on", and thus ZT2 corresponds to 2 hours after "light on" and so forth until ZT24, which corresponds to 24 hours after "light on" and 12 hours after "light off". This experiment will use a single time point at ZT6 because of the proven data in other tissues showing that ZT6 is most likely the time when BMAL1 binding to DNA is highest. This single time point should be enough to qualitatively and quantitatively identify the genes that are targeted by the molecular clock. If possible, there will be 3 mice per time point in order to show consistency with the data, minimize unnecessary sacrificing, and still be statistically valid. We will also use *Bmal1* knockout mice. These mice are deficient for the gene *Bmal1* and are molecularly and behaviorally arrhythmic and thus will be used as a control. No other variable, other than the untestable biological difference between individual mice and their genetic composition (*Bmal1* knockout or WT) will change with this experiment. To analyze the data, *Bmal1*-associated chromatin are immunoprecipitated, purified,

and the enrichment of certain genomic regions are determined by quantitative real-time PCR (qPCR). This is a method used widely in molecular biology involving the use of specific DNA primers to detect and compare the amplified DNA regions, which will be the regions of CLOCK:BMAL1 binding. For analysis of RNA gene expression, tissues are harvested every 4 hours for 24 hours (6 time points equally separated every 4 hours), and their mRNA will be extracted. The cDNA is synthetized and rhythmic gene expression is determined by qPCR using gene specific primers (see below).

CHAPTER II

MATERIALS AND METHODS

Animals

Mice housed in 12-hour light and 12-hour dark cycle (LD12:12) are used. All experiments are performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Texas A&M Institutional Animal Care and Use Committee (IACUC protocol #2013-0158).

Tissue collection

After anesthetization of the mouse at the appropriate time point with isoflurane followed by quick decapitation, the intestine is removed and cut ~1cm away from the stomach and ~1cm before the cecum. The large intestine is cut as close the anus as possible and ~1cm after the cecum. The dull end of a single edge razor is used to gently remove the feces from the lumen. The intestine is then cut longitudinally to expose the lumen and washed with a solution of ice-cold phosphate buffered saline (PBS) to gently remove feces and associated connective tissue. When preparing a whole tissue, the intestine is washed three times in ice-cold PBS solution and flash frozen in liquid nitrogen for later processing. When extracting the intestinal epithelial cells, the intestine is weighed, placed into a 5mM DTT and HBSS (0.137M NaCl, 5.4mM KCl, 0.25mM Na2HPO4, 0.1M glucose, 0.44mM KH2PO4, 1.3 mM CaCl2, 1 mM MgSO4, 4.2 mM NaHCO3) solution at a ratio of 20ml/g of tissue, placed in a 37°C incubator, and shaken vigorously for a ratio of 20min/g of tissue. This first wash with DTT is necessary to remove any remaining feces and disassociate the intestine from the mucosal layer. This layer is discarded.

The intestine is then transferred to the second solution of 5mM EDTA in HBSS-no Magnesium or Calcium (0.137M NaCl, 5.4mM KCl, 0.25mM Na2HPO4, 0.1g glucose, 0.44mM KH2PO4, 1.3 mM CaCl2) at a volume of 15ml/g of tissue for 15min/g of tissue and shaken at 37°C. Three consecutive washes of the same parameters removes the epithelial cells. The cells are strained through a 70µl strainer and centrifuged for 2 minutes at 500 x g to remove any unwanted fat or connective tissue from the pellet. Each wash fraction is centrifuged at 500 x g for 5 minutes to pellet the epithelial cells. The supernatant is removed and discarded while the pellet fractions are combined, aliquoted and flash frozen.

Total RNA extraction

Total mRNA extraction was performed using the Guanidinium thiocyanate-phenol-chloroform extraction method using Trizol (Invitrogen, Carlsbad, California). Using a cold mortar and pestle, the frozen whole intestine was crushed into a fine power and 100µl aliquated into 5 or 6 eppendorf tubes, depending on the size of the tissue. Small intestines produced ~500µl of total powder while large intestines produced ~200µl of total powder. 500µl of Trizol was added to ~100µl of frozen intestinal powder. The tissue was homogenized in 30 second intervals three times. Another 500µl of Trizol was then added, mixed, and the solution was incubated at room temperature for 5 minutes. After the incubation, 200µl of chloroform was added and shaken vigorously for 15 seconds. The solution was incubated at room temperature for 3 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. Once the solution separated, there was a bottom layer of Trizol containing denatured proteins, a middle layer of DNA, and a top aqueous layer containing RNA. 250µl of the top aqueous phase was removed without disturbing the middle layer of DNA and 250µl of isopropanol was added to precipitate total RNA, mixed well,

and incubated at room temperature for 10 minutes. After incubation, the solution was centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA was pelleted and washed with 1mL of 75% ethanol solution. After centrifuging at 7,500 x g for 5 minutes at 4°C, the ethanol was removed and the pellet was air dried to remove all traces of ethanol. The RNA pellet was resuspended in 30µl -50µl mH2O depending on the size of the pellet. The total extracted RNA concentration was determined using a Quantus™ Fluorometer (Promega) and the Quantifluor RNA system. [How much do you retrieve per tissue, approximately/range].

cDNA synthesis

Prior cDNA synthesis, total RNA was first processed for DNase treatment in order to remove any DNA contamination. To this end, 25µg of total RNA was treated with 1µl DNAse (TURBO DNAse) and 1X TURBO DNAse buffer to remove any traces of DNA in the sample. The volume of RNA was brought to 43µl using RNAse-free water and the solution was incubated at 37°C for 30 minutes in a thermocycler. Another 1µl of DNAse was added after the initial 30-minute incubation and the solution was set to incubate for another 30 minutes. Then, 10µl of DNAse inactivation reagent was added to each tube, mixed, and left to incubate at room temperature for 5 minutes. The solution was centrifuged at 10,000 x g for 5 minutes at 4°C to pellet the beads of the DNAse inactivation reagent. After centrifugation, the supernatant containing DNAse-free total RNA was removed without disturbing the pellet. To produce cDNA, 2.5-3.5µg of total RNA was reverse transcribed (be precise for each tissue) and amplified using 250ng of random primers, 1µl of dNTP, and RNAse-free water to bring the solution total volume to 16µl . A negative "No Reverse Transcriptase - (NO RT)" control was also made to assess for any DNA contamination. The mixture was heated for 5 minutes at 65°C to linearize

the RNA and quickly chilled on ice. The reverse transcriptase RNAseOUT/Superase-In (1µl), along with 4µl of 5X First Strand buffer and 2µl of 0.1M DTT, were added to the solution and incubated at 25°C for 2 minutes. One microliter of Superscript II Reverse Transcripase was added (negative control did not receive the reverse transcriptase enzyme) and incubated in the thermocycler at 25°C for 10 minutes, 42°C for 50 minutes, and 70°C for 15 minutes. By this step, the cDNA had been transcribed, so 1µl of E.coli RNAseH was added to the solution and incubated at 37°C for 20 minutes to degrade the RNA. The final solution was diluted 10X to a total volume of 200µl. To analyze gene expression using the cDNA, specific primer pairs that code for CLOCK:BMAL1 target genes were generated and used through a standard qPCR protocol (see Table 1: Primers).

Chromatin immunoprecipitation

Single cross-linking chromatin immunoprecipitation is performed using both whole intestinal tissue and isolated intestinal epithelial cells. When using the whole intestine, the tissue is flash frozen in liquid nitrogen and crushed into a powder using a cold mortar and pestle. When using only intestinal epithelial cells, the cells are either used fresh or flash frozen. The crushed intestinal powder or epithelial cells are transferred into a dounce homogenizer and cross-linked with 4ml of 1X PBS and 1% formaldehyde for 10 minutes at room temperature. The tissue is homogenized and 70µl of 2M glycine is added to stop cross-linking. The solution is equally aliquoted into four 2ml eppendorf tubes and washed twice with 1ml of hypotonic buffer (10mM Hepes pH 7.6, 15mM KCl, 15% NP-40, 1mM DTT, and 1mM PMSF). The sample is resuspended in 500µl of hypotonic buffer and added to a dense sucrose cushion (10mM Hepes pH 7.6, 15mM KCl, .15% NP-40, 24% sucrose, 1M DTT, and 1mM PMSF) and centrifuged at

20,000 x g for 10 minutes at 4°C. The pellet at the bottom of the tube is removed and washed four times with 1ml resuspension buffer (1mM Tris pH 7.5, 150mM NaCl, 2mM EDTA, 1mM PMSF). Three aliquots in 500µl of a sonication solution (10mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, .25% SDS, .2% Triton, 1X Protease Inhibitor Cocktail) are used for the intestines. The samples are sonicated for 6 cycles of 15 seconds "on" and 105 seconds "off". When sonicating, the nuclei are kept on ice-cold ethanol for the duration of the sonication and placed on ice during the "off" period. The samples are spun at 15,000 x g for 10 minutes at 4°C in order to pellet the junk. The supernatant is removed without removing any of the pellets and placed in a new eppendorf tube. A 25µl sample is removed as an input standard at this step (see below). The rest of the sample is diluted to a standard concentration of 1% Triton, .1% Na deoxycholate, .1% SDS, 150mM NaCl, 10mM Tris pH 7.5, 2mM EDTA. One microliter of BMAL1 specific antibody (Santa Cruz, Rabbit Ab to BMAL1, Lot# GR198879-8) is added and rotated in the diluted chromatin overnight at 4°C. 25µl of Dynabeads Protein G are washed twice with Final IP Buffer (10mM Tris pH 7.5, 150mM NaCl, 2mM EDTA, .1% Na Deoxycholate, 1% Triton X-100, and 1X Protease Inhibitor Cocktail). After washing the Dynabeads, the solution is resuspended in 1mL Blocking Solution (Final IP buffer, .1 mg/ml yeast tRNA, 1 mg/ml BSA) and rotated separately overnight at 4°C.

The next day, the Dynabeads are washed again with 1ml Final IP buffer using a magnetic strip. The chromatin is added to the Dynabeads and rotated at 4°C for two hours. Nine washes followed with a 10 minute rotation time at 4°C between each wash. The chromatin is washed twice with TSE I (10mM Tris pH 7.5, .1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl, 1mM DTT, 1X Protease Inhibitor Cocktail), twice with TSE II (10 mM Tris pH 7.5, .1% SDS,

1% Triton X-100, 2mM EDTA, 500mM NaCl, 1mM DTT, 1 mM PMSF), twice at TSE III (10mM Tris pH 7.5, .25 M LiCl, 1% NP-40, 1% Na deoxycholate, 1mM EDTA, 1mM DTT, 1mM PMSF), twice with TEN+ Triton (10mM Tris pH 7.5, 1mM EDTA, 150mM NaCl, .1% Triton), and once with TE+ Triton (1X TE, .1% Triton). After washing, the Dynabeads are resuspended in 200µl of ChIP Elution Buffer (50mM Tris-HCl pH 8.0, 10mM EDTA, 1% SDS, 1mM DTT), while the 25µl of input collected earlier is resuspended in 175µl of ChIP Elution buffer. The beads are incubated at 65°C for 6-18 hours. After incubation, the solution is removed from the Dynabeads because the chromatin had been eluted from the beads into the solution. The supernatant is put in a new tube and diluted by adding 200µl of 1X TE in order to dilute the SDS. On the RNAse bench, 8µl of 1mg/mL RNAse is added to the diluted samples and incubated at 37°C for 30 minutes. Next, 2µl of 20mg/mL Proteinase K is added to the samples and incubated at 55°C for one hour. The chromatin is aliquoted into two 1.5 mL eppendorf tubes and washed with 1mL of PB Binding Buffer provided by a Qiagen DNA purification kit. The solution ran through a purification column and a Qiagen column vacuum and the columns are washed with 750µl of PE Buffer. After the wash, the columns are centrifuged at 11,000 x g for 2 minutes in an RNAse free centrifuge. The remaining top half of the column is placed on top of a new tube and washed with 40µl of Qiagen Elution Buffer, centrifuged again, and the final solution enrichment is verified via qPCR.

qPCR

Samples used for the quantitative PCR (qPCR) protocol are first diluted, with a dilution of the ChIP samples by ½ and a dilution of the Input samples by 1/10 using 1X TE. A standard curve using a mixture of all the ChIP samples is also made in order to test for the efficiency/quality of

the primers. Specific *Bmal1* target sites in the Dbp gene were used (see Table 1: Primers) to detect the extent to which BMAL1 is bound to chromatin. An intergenic region in the Dbp gene was also used to set the negative control.A master mix was made consisting of 500nM of each primer (forward and reverse), 1X of fluorescent dye (2X iTaq Universal SYBR Green Supermix, Bio-Rad) and mH_2O. 2µl of each sample was mixed with 20µl of the master mix. Of the total 22µl, 9.8µl was taken from each sample twice and individually placed into two wells of a 96-well qPCR plate to create duplicates. The fluorescent dye (SYBR Green) binds to the DNA and provides a measurable fluorescence signal as DNA is synthetized. The 40 cycles were set at 95°C for 10 minutes, 59.5°C for 20 minutes, 72°C for 30 minutes with a final 5 minutes at 65°C and 30 seconds at 95°C. A melting curve was generated to assess for the specificity of the primer pair and amplification. The melting curve measures the specific melting point of the DNA, ideally producing one peak. Different stands of DNA melt at slightly different temperatures based on their size and sequence, so the melting curve was used to measure any contamination.

Table 1: Primers

Gene	**Sequence**
Bmal1 Forward	5'- ATGCTCACACGGTGCAGACA- 3'
Bmal1 Reverse	5'- CTGCTCAGGCACATTCCTCAT -3'
Rev-era Forward	5'- TGAATGACCGCTTTCAGCTG- 3'
Rev-era Reverse	5'- CACTAGAGCCAATGTAGGTG- 3'
Aldob Forward	5'- TGTTATCATTAACCCAGCTTGC-3'
Aldob Reverse	5' –CTGCCACCTCACACAGCTT- 3'
Dbp1st Intron Forward	5'- ATGCTCACACGGTGCAGACA- 3'
Dbp 1st Intron Reverse	5'- CTGCTCAGGCACATTCCTCAT-3'
Villin Forward	5'- CTGAATGCCCAAGTCAAAGG-3'
Villin Reverse	5'-CGAAGAAGCTTCCAAAGGTG-3'
Intergenic Forward	5'- CTTTTAATGAGGCTGTGTGGA-3'
Intergenic Reverse	5'- ACTCCCTTGCGAATGTCCTA-3'

CHAPTER IV

RESULTS

Analysis of rhythmic gene expression in whole intestinal tissue

In order to look at rhythmic gene expression in the mouse intestine, we collect both whole small intestine and whole large intestine from wild-type mice at six different time points throughout the day, taking 2 animals per time point. Tissues are cut longitudinally to expose the lumen, washed with 1X PBS to remove feces/bacteria and connective tissue, and flash-frozen in liquid nitrogen (see methods for more details). Total RNA is extracted from frozen tissues using Trizol and the RNA concentration is determined using a ssRNA Quantifluor assay kit from Promega. The total amount of RNA recovered is between 250µg and 500µg for the small intestine, and between 75µg and 100µg for the large intestine.

To generate cDNA from total RNA, 25µg of RNA is first treated with DNAse to remove any possible contamination from genomic DNA. Then, total RNA quality and integrity is assessed by gel electrophoresis on a 1% Agarose and 1X TAE gel using a 1X TAE buffer (Figure 1). The clean and distinguishable bands, corresponding to 28S and 18S rRNA, indicate high quality RNA with little to no degradation. The cDNA is synthesized with Reverse Transcriptase using 2.5µg of RNA. We used qualitative real-time polymerase chain reaction (qPCR) to determine the levels of mRNA expression of a few clock genes throughout the day. The qPCR machine quantifies the amplification of target genes during PCR using fluorescent dyes. In both the whole small and whole large intestines, *Rev-erba* expression is rhythmic with peaks of expression at ZT6-ZT10. *Bmal1* expression is also rhythmic, but out-of-phase compared to *Rev-erba*

expression, as expression levels are minimal at ZT10-ZT14 (Figure 2 and 3). Importantly, little variation is observed between animals at the same time points. Our data thus indicate that clock genes are rhythmically expressed in the small and large intestine, suggesting that the activity and function of intestinal tissues oscillates throughout the 24-hr day.

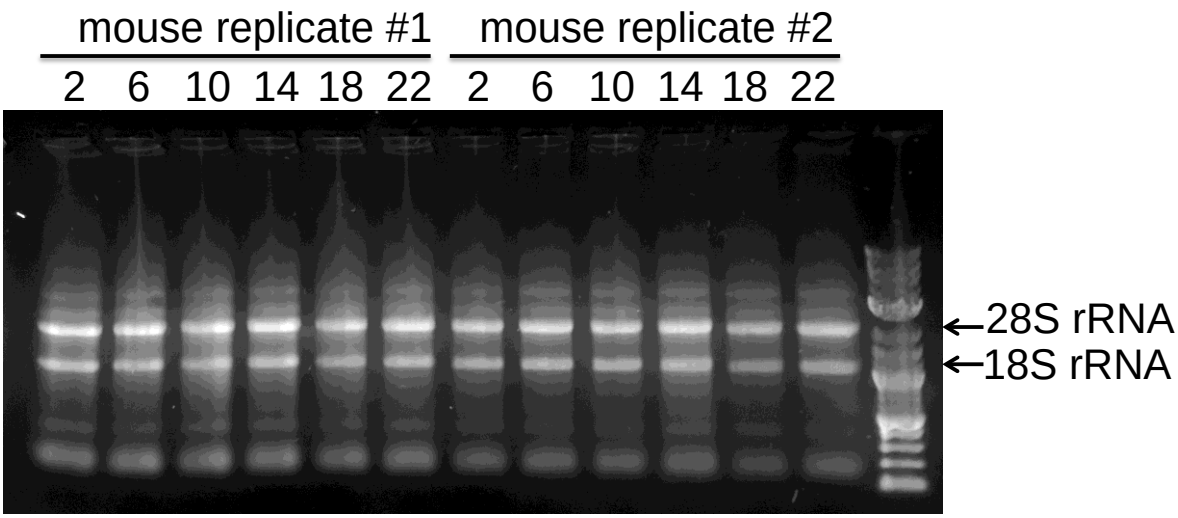

Figure 1: Gel electrophoresis of total RNA extracted from the whole mouse large intestine. Total RNA is extracted from large intestine collected from mice sacrificed at different times of the day (2, 6, 10, 14, 18 or 22 hours after light on). Two independent mice are sacrificed for each time point. The gel electrophoresis profile shows that total RNA is not degraded, as both 28S and 18S rRNA yielded to sharp and bright bands.

Figure 2: Gene expression of *Rev-erba* and Bmal1 using mRNA from the whole large intestine. Two biological wild-type replicates are used. The data show that the clock genes *Rev-erba* and *Bmal1* are both expressed rhythmically in the mouse large intestine, although with different phase of expression.

Analysis of gene expression in purified intestinal epithelial cells

Since the intestinal tissue is composed of different layers of cells including a mucous layer, an epithelial layer, two smooth muscle layers, and also contains immune cells, it is very likely that these cells subserve different functions, and thus exhibit different levels of gene expression that are differentially influenced by the molecular clock. In order to look more specifically at a subset of these cells that regulate absorption, nutrient intake, and infection, we aimed at isolating the epithelial cells of the intestine and analyze gene expression levels within purified intestinal epithelial cells. To this end, we used previously published protocols (9) to set up the procedure in

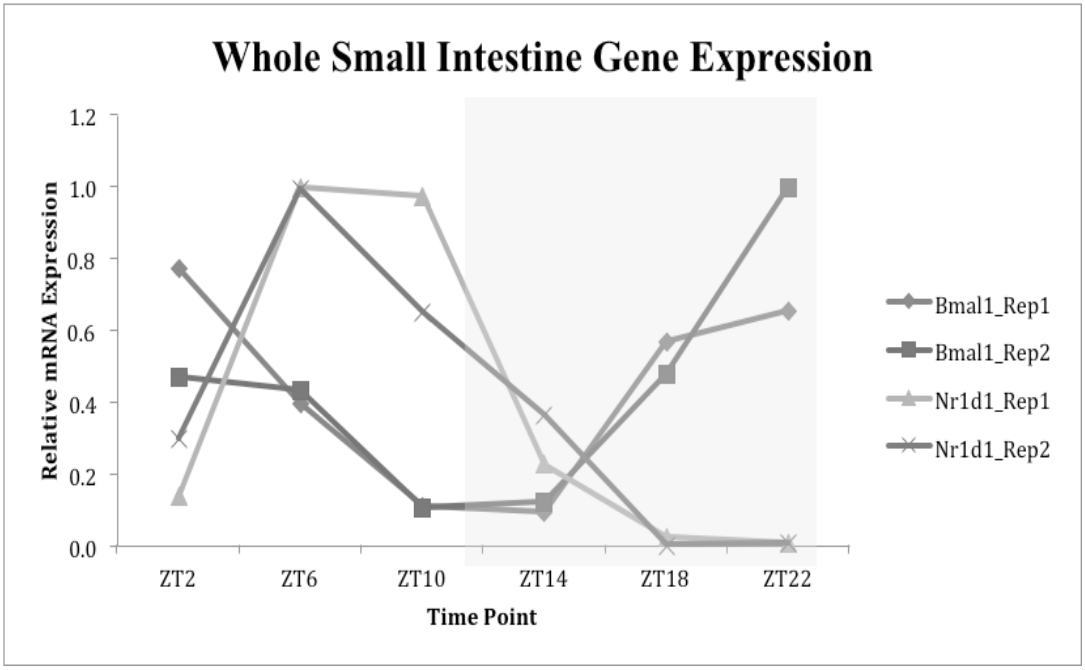

Figure 3: Gene Expression using mRNA from the whole small intestine. Two biological wild-type replicates are used and show a consistent in-phase rhythm for the clock gene Rev-era and a consistent out-of-phase rhythm for the clock gene Bmal1 similar to that seen with the large intestine. The gray area represents the dark cycle after ZT12, which is when mice are active.

the lab, using intestines collected from wild-type mice in the middle of the day (ZT6). The intestine is cut longitudinally to expose the lumen and washed with 1X PBS to remove feces/bacteria. However, instead of flash freezing the tissue in liquid nitrogen, the tissue is processed fresh to extract the epithelial cells. The tissue is washed once with a solution of HBSS and 5mM DTT to remove the mucous layer and three times with HBSS and 5mM EDTA (see methods for more detail) to dissociate the intestinal epithelial cells from the muscle layers and immune cells. Dissociated cells are then washed in the same manner as nuclei, starting with the hypotonic wash buffer (see methods for details). The cells are strained through a 70µl strainer

and centrifuged for 2 minutes at 500 x g to remove any unwanted fat or connective tissue from the pellet. About 500µl of epithelial cells total are collected from each small intestine and ~75µl of cells total from each large intestine. The large difference in the amount of recovered cells reflects the size difference between the small and large intestine in the mouse.

Similarly to the procedure with whole intestinal tissues, we performed total RNA extraction, DNAse treatment, and Reverse Transcription treatment to generate cDNA from IECs. Similarly to the whole tissues, total RNA extracted from intestinal epithelial cells yielded to clean RNA with no sign of degradation as well as the whole intestine stripped of IEC (Figure 4). To examine the purity of collected epithelial cells, we compared by qPCR the mRNA expression levels of the intestinal epithelial cell marker gene, *Villin*, to the expression of *Villin* in the whole small intestine tissue stripped of IECs. (Figure 5). This procedure was not performed on the large intestine. The data show that the IEC marker gene in the epithelial cells is expressed at higher levels as compared to the whole stripped intestine. Specifically with the wash fractions, the second fraction collected in both replicates shows the highest level of expression for the IEC marker *Villin*. Further testing is however required to validate this information through smooth muscle cell markers.

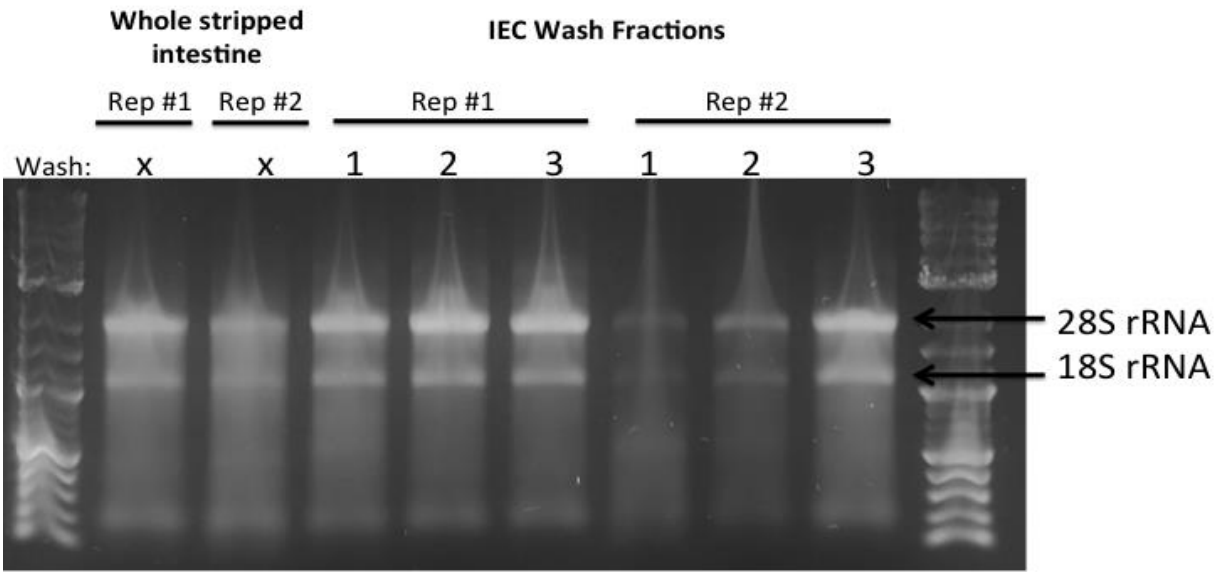

Figure 4: Gel electrophoresis of total RNA extracted from the epithelial cells of the mouse small intestine. Total RNA was extracted from IEC purified from small intestine of mice sacrificed at ZT6. The gel electrophoresis profile shows that total RNA is not degraded, as both 28S and 18S rRNA yielded to sharp and bright bands. The tail produced from this picture is due to different levels of buffer not reaching the top of the gel, delaying the electrophoretic migration of some of the sample. The tail does not reflect degradation of the RNA.

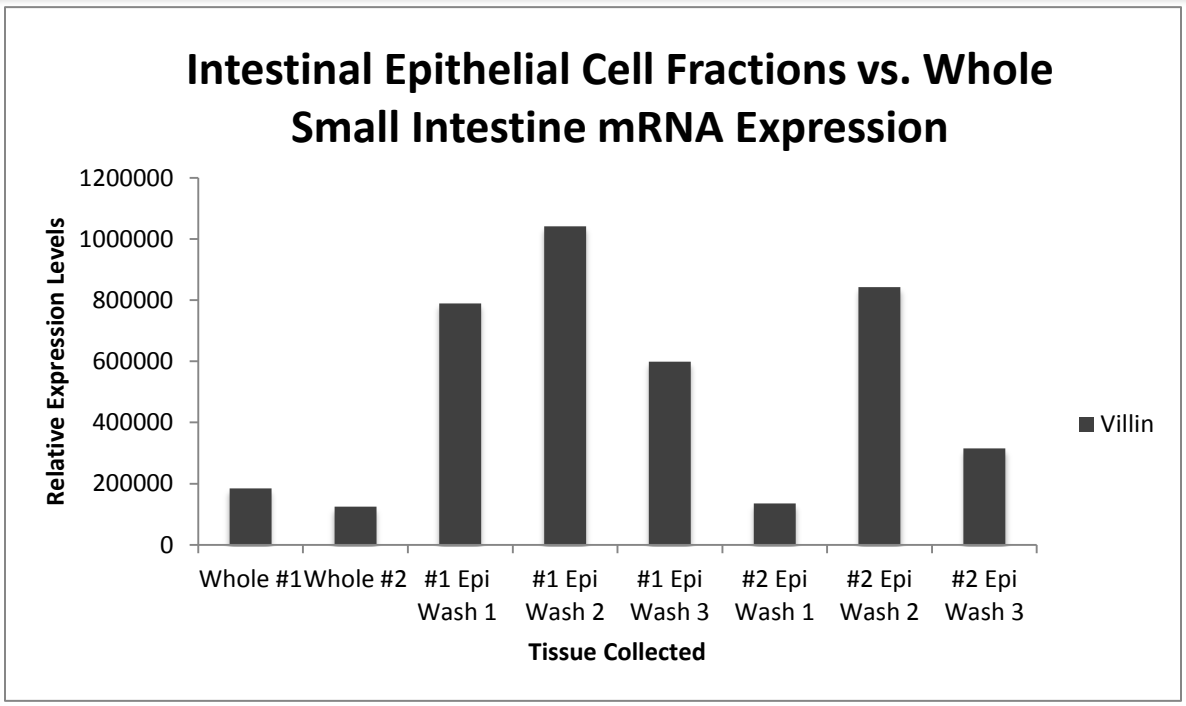

Figure 5: Intestinal Epithelial Cell mRNA expression using two wild-type mice replicates (#1 and #2). All tissues were collected at ZT6 and each IEC wash was kept separate (3 washes total each) while the whole small intestine stripped of epithelial cells was also processed after the treatment. The gene *Villin* is used as an intestinal epithelial cell marker. The stripped intestines show lower expression of *Villin* for both replicates, while the washes show higher levels of expression, especially the second wash.

Analysis of BMAL1 and RNA Polymerase II DNA binding in purified intestinal epithelial cells by chromatin immunoprecipitation

To start gaining insight into the mechanisms by which the molecular circadian clock regulates rhythmic gene expression in the intestine and its epithelial cells, we set up a protocol to perform chromatin immunoprecipitation (ChIP) in purified intestinal epithelial cells. To this end, we collected fresh intestine tissue from mice sacrificed at ZT6, stripped off the epithelial cells, and cross-linked them with 4 ml of 1% formaldehyde for 10 minutes at room temperature before

flash freezing the tissue. This ensures that the freezing and thawing process does not have an effect on the binding levels of proteins to DNA, which produces better fold enrichment results.

To determine if the purification, crosslinking and sonication procedures do not impede on the detection of proteins bound to the DNA, we first examined the binding efficiency of RNA polymerase II (Pol II) at a genomic location presumed to exhibit strong Pol II ChIP signal (transcriptional start site of a gene highly expressed in intestinal epithelial cells: *Aldob*) and at a genomic location where Pol II is not supposed to be recruited (intergenic region). Our results show a consistently higher binding of Pol II to the DNA at *Aldob* TSS compared to the base line intergenic region, where no Pol II is expected to be recruited (Figure 7). The fold enrichment, or difference between the base line Intergenic and the expressed gene is between 16X to over 30X.

One ultimate goal of my project is to characterize the genes that are directly targeted by the genes CLOCK and BMAL1 in intestinal epithelial cells. To start addressing this question, we performed a ChIP using a BMAL1 antibody within these cells. Using a similar strategy as above, we found that BMAL1 was recruited to the 1st intron of the clock gene *Dbp*, but not to the control intergenic region (Figure 8). However, the fold enrichment is lower than for Pol II, and between 10X and 20X. Although more testing is needed to confirm these results, our data indicate that our protocol enables the detection of both Pol II and BMAL1 DNA binding sites in the mouse intestinal epithelial cells.

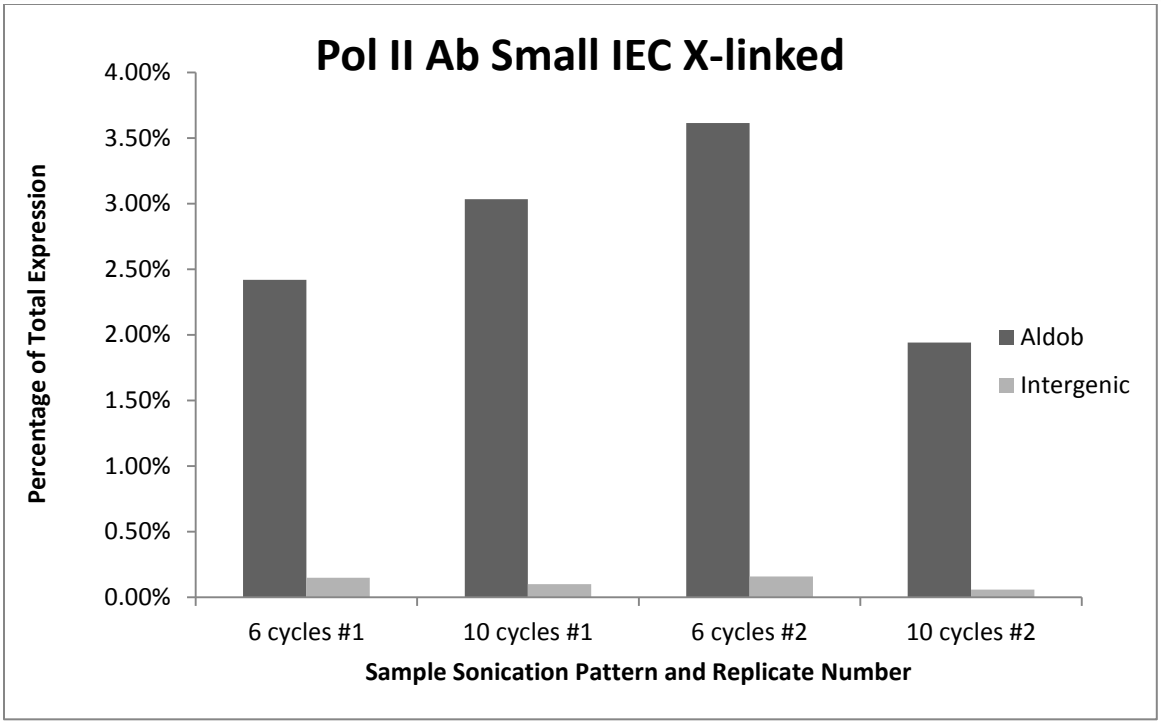

Figure 7: Intestinal epithelial tissue cross-linked with 1% formaldehyde for 10 minutes at room temperature. Two wild-type biological replicates (#1 and #2) were used and the IECs are extracted using the method from the "Journal of Immunology". The DNA is sheered using a sonicator at different cycles of 15 seconds ON and 1:45 seconds OFF at a consistent power. The higher the number of cycles indicates a higher amount of DNA sheering. The data indicates a higher level of expression for the *Aldob* gene vs. intergenic regions when selecting for the RNA Polymerase II protein on the DNA with fold enrichment between 16X and 30X.

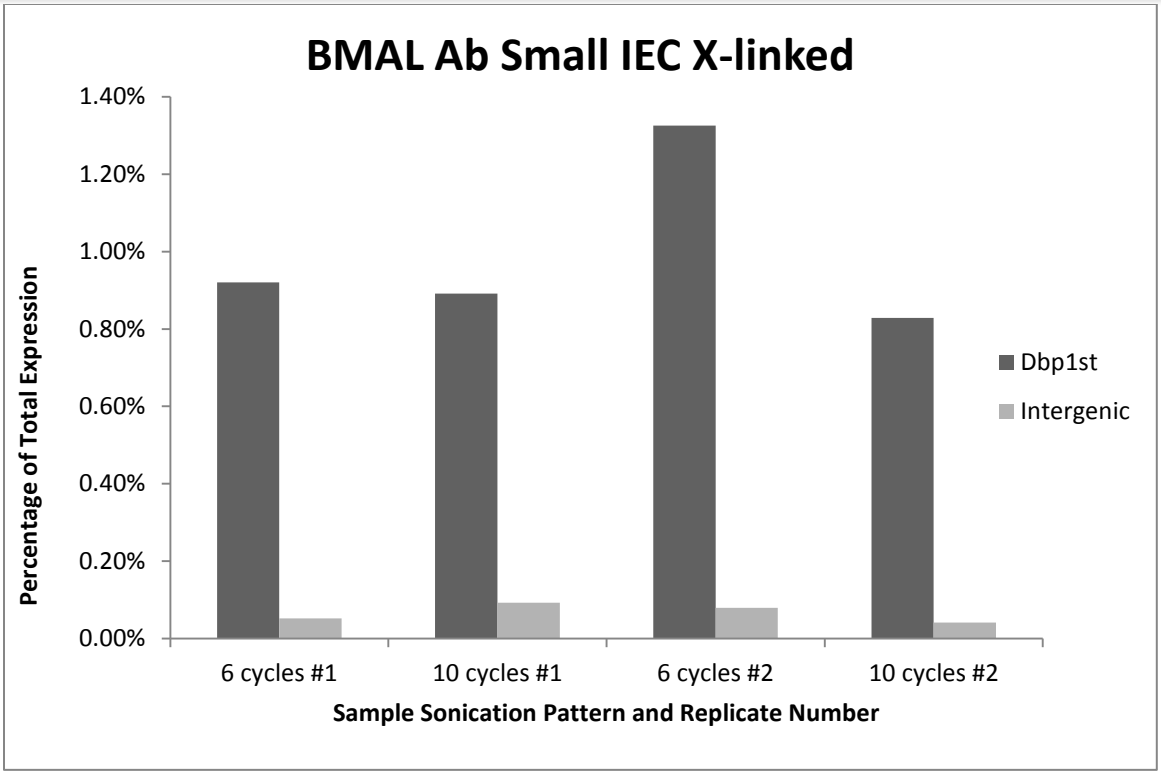

Figure 8: Intestinal epithelial tissue cross-linked with 1% formaldehyde for 10 minutes at room temperature. Two wild-type biological replicates (#1 and #2) were used and the IECs are extracted using the method from the "Journal of Immunology". The DNA is sheered using a sonicator at different cycles of 15 seconds ON and 1:45 seconds OFF at a consistent power. The higher the number of cycles indicates a higher amount of DNA sheering. The data indicates a higher level of expression for the *Bmal1* gene vs. intergenic regions when selecting for the BMAL1 protein on the DNA with fold enrichment between 10X and 20X.

CHAPTER V

CONCLUSION

In my project, I set up techniques to efficiently determine rhythmicity in the intestine through RNA extraction and mRNA analysis using quantitative PCR. I also set up ChIP techniques that will allow us to analyze the genes targeted by CLOCK:BMAL1. These protocols will be used in the future to characterize the genes that are rhythmically expressed in different regions of the intestinal tract through quantitative real-time PCR as we have with other peripheral tissues, like the heart, kidney, and liver. Completion of my project has enabled the lab to examine more precisely how the circadian clock controls the rhythmic digestion in mammals, and represents a strong foundation for further studies that will examine the role of rhythmic feeding behavior in the biology of the intestine.

As stated above, the extraction of RNA from the small intestine and large intestine with sharp rRNA bands indicates our ability to extract RNA from these tissues with little to no RNA degradation. Using these samples collected at 4-hour time points, we were able to show rhythmicity through oscillation of the *Bmal1* gene as well as another core clock gene, *Rev-erba*. This was a good first step indicating that the intestine biology is, similarly to other peripheral tissues, under the control of the circadian clock. Although expected, it was a crucial first step because it provides our lab with data showing our ability to successfully extract and analyze different subsections of RNA from the intestinal tissue. In both the large and small intestine, mouse replicates show a similar pattern of gene expression, where the mRNA that codes for *Rev-erba*, a key regulatory component in the circadian clock, is rhythmically expressed with a peak

phase at ZT6 while *Bmal1* peaks at ZT22. Interestingly, the phase of *Rev-erba* and *Bmal1* mRNA expression are similar to those observed in other peripheral tissues such as in the liver (10).

Using the ChIP method on the whole small and large intestine was generally unsuccessful because it generated a lot of background and producing low fold enrichment. We thus decided to perform the ChIP on purified mouse intestinal epithelial cells, which provided much higher fold enrichment using both the BMAL1 antibody and Pol II antibody. With the purified IEC, freezing the cells after extraction and then thawing, cross-linking, and proceeding with the ChIP showed a low fold enrichment, so we began the method of cross-linking the cells with 1% formaldehyde before storing the cells, thus increasing the fold enrichment. The ChIP on the mouse IEC collected at ZT6 (middle of the day) showed high fold enrichment around 10X-20X when using the BMAL1 antibody and an even higher fold enrichment of 20X-30X with the Pol II antibody. These results indicate that we are now able to perform ChIP experiments on intestinal tissues to a degree where sequencing the DNA fragments immunoprecipitated by either BMAL1 or Pol II would be an option. This would allow us to further analyze the tissue specific gene expression and further show that the intestine is a peripheral tissue expressing rhythmicity in the same manner that has been shown in our lab with the heart, kidney, and lungs.

Finally, we have been able to extract high-quality total RNA from the mouse intestinal epithelial cells. We verified the purity of the collected IECs by testing the expression of an IEC cell marker, *Villin,* and compared the levels of mRNA expression of the whole small intestine after being stripped of cells to each IEC wash fraction. The expression of *Villin* was lowest in the

whole intestine, as expected, and the level of *Villin* expression in the fractions of IEC washes, especially the second wash, was much higher. Although more validation is required by testing smooth muscle cell markers as well as housekeeping genes, this provides a strong foundation and gives us confidence that the cells extracted are actually epithelial cells.

Completing this project indicates that clock genes are rhythmically expressed in the intestine, and that we are able to perform ChIP to further investigate the underlying mechanisms. This will enable us to move forward confidently on other experiments and explore, for example, the effects of the micobiome on the intestinal circadian rhythm or experiments that affect the feeding behavior of the mice.

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